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3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Inhibitors (Statins) Induce Hepatic Expression of the Phospholipid Translocase *mdr2* in Rats

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Background & Aims: Biliary cholesterol secretion is coupled to that of phospholipids in a process controlled by *mdr2* P-glycoprotein activity and bile salt secretion. Statins, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have been shown to affect hepatobiliary lipid secretion in rats. The aim of this study was to relate the effects of statins on bile formation to the expression of *mdr2* and other hepatic adenosine triphosphate-dependent transport proteins involved in bile formation in rats. **Methods:** Rats received simvastatin- or pravastatin-containing chow continuously for 5 days. In one group of rats, simvastatin treatment was withdrawn 9–12 hours before the end of the experiment to induce biliary cholesterol hypersecretion (rebound). Bile and liver tissue were collected for lipid analysis, and hepatic messenger RNA (mRNA) and protein levels were studied by reverse-transcription polymerase chain reaction, immunoblotting, and immunohistochemistry. **Results:** Simvastatin feeding did not alter biliary bile salt secretion. Secretion of phospholipids and cholesterol was stimulated by 74% and 90%, respectively, in the simvastatin-continuous group and by 72% and 235%, respectively, in the rebound group compared with controls. *mdr2* mRNA levels increased only in the continuous group. *mdr2* protein levels increased in both simvastatin-fed groups. Induction was most pronounced in periportal hepatocytes. *mdr1b* mRNA levels were moderately increased in both simvastatin-fed groups. Levels of other hepatic transport proteins did not change. Similar results were obtained in pravastatin-fed rats. **Conclusions:** Statins increase expression of *mdr2* and *mdr1b* in rats, revealing a novel effect of these commonly used drugs.

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis.¹ These drugs are widely used in the treatment of hypercholesterolemic

patients.² Statins lower plasma low-density lipoprotein (LDL) cholesterol levels in humans by (1) enhanced clearance of LDL by transcriptional up-regulation of hepatic LDL receptors and/or (2) reduced production of LDL by either decreasing its secretion into the plasma or by decreasing the conversion of very-low-density lipoprotein to LDL.^{3,4} Statins do not lower plasma cholesterol levels in rodents that carry most of their plasma cholesterol in the high-density lipoprotein fraction.⁵ Yet statins have been shown to affect hepatobiliary lipid secretion in rats. Long-term (>5 days) statin treatment results in induction of hepatic HMG-CoA reductase expression and enzyme levels and promotes biliary lipid output.^{6,7} Additionally, cessation of statin treatment leads to marked hypersecretion of cholesterol into bile within 6–12 hours,^{8,9} accompanied by a relatively smaller increase in biliary phospholipid secretion.⁹ Bile salt secretion, on the other hand, is not influenced.^{6,8,9} Because both cholesterol and phospholipid secretion are coupled to that of bile salts,¹⁰ the statin-induced secretion of lipids indicates that these drugs exert specific actions in the liver. The hypersecretion of cholesterol into bile after statin withdrawal has been attributed to an inappropriately high hepatic cholesterol synthesis because of induction of the HMG-CoA reductase system.⁸ Evidently, this does not explain the concomitant hypersecretion of phospholipids.

Abbreviations used in this paper: ABC, adenosine triphosphate-binding cassette; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; MDR, multidrug resistance; MRP, multidrug-resistance protein; *mdr*, *mrp*, genes encoding multidrug-resistance proteins; PC, pravastatin-continuous; PCR, polymerase chain reaction; Pgp, P-glycoprotein; SC, simvastatin-continuous; spgp, sister of Pgp; SR, simvastatin-rebound; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein.

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Recently, it has become clear that phospholipid secretion into bile is controlled by *mdr2* P-glycoprotein (Pgp) in the bile canalicular membrane.¹¹ This *mdr2*-Pgp is a member of the Pgp subfamily of adenosine triphosphate-binding cassette (ABC) transport proteins, which in rodents comprises at least 4 members that are all localized in the canalicular domain of hepatocytes: *mdr1a*, *mdr1b*, *mdr2*, and *spgp*. Expression of *mdr2* in normal rodent liver is high, and evidence indicates that this protein functions as a flippase that translocates phosphatidylcholine across the membrane.¹¹ In contrast to *mdr2*, *mdr1a* and *mdr1b* are present at low levels in normal rat liver.¹² Overexpression of the latter 2 proteins in cells confers multidrug resistance against a broad variety of natural product drugs.^{13,14} The physiological function of *mdr1a* and *mdr1b* in the liver presumably involves hepatobiliary transport of bulky amphiphilic compounds, including hydrophobic peptides, steroids, and, perhaps, certain lipids.^{14–16} *Spgp*, the product of the so-called sister gene of Pgp, is abundantly present in rat canalicular membranes and was recently shown to function as the major bile salt export pump.^{17,18}

The multidrug-resistance protein (MRP1) and its isoforms are members of another subfamily (MRP/CFTR) of the ABC protein superfamily.^{15,19} The human MRP1 and its rat homologue *mrp1* are expressed at low levels in the normal liver.^{20,21} The recently cloned *mrp1* homologue *mrp2*^{22,23} has a similar substrate specificity as *mrp1* and is highly expressed in the canalicular membrane domain of hepatocytes and functions as a canalicular multispecific organic anion transporter.^{24,25} *Mrp2* has been implicated in the removal of pravastatin from the body by mediating its secretion into bile.²⁶

To gain insight in the mechanism(s) underlying statin-associated changes in biliary lipid secretion, we have evaluated the effects of continuous and discontinuous administration of simvastatin on the expression of genes encoding hepatic ABC transport proteins. For comparison, rats were continuously exposed to pravastatin or to diosgenin, another established inducer of cholesterol hypersecretion.^{27,28}

Materials and Methods

Animals

Pathogen-free male Wistar rats (270–310 g) were purchased from Harlan (Zeist, Netherlands). They were housed at the Central Animal Facility of the University of Groningen in a temperature-controlled environment with alternating 12-hour light (6 AM to 6 PM) and dark cycles. All experiments were performed between 7 and 9 AM. The rats received standard laboratory chow (RMH-B; Hope Farms BV, Woerden, Netherlands) and had free access to food and water. The study was

approved by the local committee for care and use of laboratory animals.

Experimental Design

Three independent animal experiments were performed.

Experiment A. Rats were fed for 5 days with either the standard control diet (control, *n* = 10) or the same diet supplemented with 0.1% (wt/wt) simvastatin (Merck, Sharp & Dome, Whitehouse Station, NJ). At the end of the 5-day period, one of the simvastatin-supplemented groups was returned to the control diet 9–11 hours before the end of the experiment (simvastatin-rebound; SR group, *n* = 10) and the other group was maintained on simvastatin-supplemented diet (simvastatin-continuous; SC group, *n* = 10). On the morning of day 6, rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally). Of each group, 3 animals were subjected to bile duct catheterization to allow collection of bile.²⁹ After a 5-minute stabilization period, bile was collected for 30 minutes and stored at –20°C for further analysis. The livers were perfused with phosphate-buffered saline (PBS), excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen, and stored at –80°C until use for isolation of membranes or of RNA. For immunohistochemistry study, small pieces of liver were frozen in isopentane and stored at –80°C until further use. Blood samples were collected in EDTA-containing tubes and centrifuged immediately (10,000*g*), and plasma was stored at –20°C until used.

Experiment B. Experiment A was repeated as described, except that simvastatin was replaced by pravastatin (Bristol-Myers Squibb, Princeton, NJ), and that a “rebound” group was omitted. Rats were fed for 5 days with either the control diet (control, *n* = 3) or the same diet supplemented with 0.1% (wt/wt) pravastatin (pravastatin-continuous; PC group, *n* = 5). At the end of the 5 days, bile was sampled, the rats were killed, and tissue was collected as described for the rats in the experiment with simvastatin.

Experiment C. Rats were fed for 5 days with either the standard control diet (control, *n* = 3) or the same diet supplemented with 0.5% (wt/wt) diosgenin, a plant saponin (Sigma Chemical Co., St. Louis, MO) (diosgenin, *n* = 3). At the end of the 5 days, bile was sampled, the rats were killed, and tissue was collected as described for the rats in experiment A with simvastatin.

Antibodies

Mouse monoclonal antibody C219 (Signet Laboratories, Dedham, MA) was used for detection of all Pgps. This antibody recognizes a conserved epitope close to the ABC in all known members of the Pgp subfamily.^{30,31} Polyclonal antibodies against *mdr2* (k111) were raised by immunizing rabbits with the peptide EEFEVELSDEKA coupled to keyhole limpet hemocyanin (KLH) via the NH₂-terminus.³² k111 was used for the detection of *mdr2* on immunoblots. Mouse monoclonal antibody P₃II-26, raised against a specific sequence of the human MDR3³³ (kindly provided by Dr. R. J. Scheper, Free

University Hospital, Amsterdam, Netherlands), was used for immunohistochemical detection of rat *mdr2* Pgp. On Western blot, k111 and P₃II-26 recognize a 170-kilodalton protein in canalicular membrane-enriched fractions from rat liver but not in basolateral membrane-enriched fractions. In addition, in membrane subfractions from insect cells infected with a recombinant baculovirus expressing the rat *mdr2* gene, a single band was observed, whereas no specific signals were detected in membranes from cells overexpressing the rat *mdr1b*, human *MDR1*, or human *MRP1* genes (data not shown). Polyclonal antibodies against spgp (k12) and mrp2 (k4) were raised in our laboratory.²¹

mRNA Analysis by Reverse-Transcription PCR

Total RNA was isolated from frozen rat liver using TRIzol Reagent (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. Subsequently, mRNA was isolated using the Oligotex mRNA Mini-kit (Qiagen GmbH, Hilden, Germany). Single-stranded complementary DNA (cDNA) was synthesized from 2.5 µg mRNA and subsequently subjected to relative polymerase chain reactions (PCRs) as described by our laboratory.²¹ Specific primer sets for the various hepatic ABC proteins were designed; for each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal number of cycles to be used. This was determined to be halfway through the exponential phase. For every PCR reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The number of cycles was 22 for GAPDH and mrp2, 24 for spgp, 29 for *mdr1b* and *mdr2*, and 33 for *mdr1a* and mrp1. The primer sequences and product sizes have been described previously.²¹ Sequence analysis confirmed the specificity of the PCR primers for each PCR product. In each experiment, water was used as a negative control. Ten microliters of PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using a charge-coupled device video camera of the ImageMaster VDS system (Pharmacia, Uppsala, Sweden).

Isolation of Membranes From Liver Homogenates

Crude liver cell membrane fractions and canalicular membrane-enriched fractions were isolated by the sucrose gradient centrifugation techniques described by Meier and Boyer,³⁴ with some modifications as detailed by Vos et al.²¹ For later use, membrane fractions were stored at -80°C in 10 mmol/L Tris-HCl (pH 7.4) with Complete protease inhibitor cocktail (1:50, wt/vol; Boehringer Mannheim GmbH, Mannheim, Germany).

Western Blot Analysis

The crude cell membrane proteins (100 µg) or canalicular membrane-enriched proteins (15 µg) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Amersham International, Buckinghamshire, England), using a tankblotting system

according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). BDH molecular weight standards (42,700–200,000-dalton range, BDH Ltd., Dorset, England) were used as marker proteins. The blots were stained with Ponceau S solution (0.1% Ponceau S [wt/vol] in 5% acetic acid [vol/vol]; Sigma) to confirm similar protein concentrations in every lane. The blots were incubated with the first antibody diluted in PBS containing 4% nonfat dried milk powder (Fluka BioChemica, Buchs, Switzerland) and 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma); washed in PBS/0.05% Tween 20; incubated with horseradish peroxidase-labeled swine anti-rabbit immunoglobulin (Ig) G or rabbit anti-mouse IgG (dilution 1:2000; Dako A/S, Glostrup, Denmark); and finally developed using Pierce SuperSignal Chemiluminescent Substrate Luminol/Enhancer (Pierce, Rockford, IL).

Immunohistology

For immunohistological studies, 4-µm sections were cut from frozen liver tissue. After drying, the sections were fixed in acetone for 10 minutes at room temperature. The monoclonal antibodies C219 and P₃II-26 and the polyclonal antibody k12 were diluted (1:50, 1:250, and 1:50, respectively) in PBS containing 1% bovine serum albumin. Sections were incubated with 50 µL of the diluted antibody for 60 minutes. After incubation with the first antibody, endogenous peroxidase activity was blocked by incubating the sections in 50 mL PBS containing 125 µL of a 30% H₂O₂ solution for 30 minutes at room temperature. Slides incubated with the monoclonal antibodies C219 and P₃II-26 were subsequently incubated with peroxidase-conjugated rabbit anti-mouse IgG (dilution, 1:50) for 30 minutes followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:50) for another 30 minutes. Slides incubated with the polyclonal antibody k12 were subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:100) for 30 minutes followed by incubation with peroxidase-conjugated rabbit anti-goat IgG (dilution 1:100) for another 30 minutes. All peroxidase-conjugated immunoglobulins were diluted in PBS containing 1% bovine serum albumin and 5% normal rat serum. Peroxidase activity was developed with 3-amino-9-ethylcarbazole solution (0.2 mg/mL sodium acetate buffer containing 0.03% H₂O₂) for 15 minutes at room temperature. Nuclear counterstaining was performed with hematoxylin, and the slides were covered with Kaiser's glycerin-gelatin. All antibody incubations were performed at room temperature; after each incubation, sections were rinsed with PBS.

Analytical Procedures

Protein concentrations were determined with the DC Protein Assay (Bio-Rad) using bovine serum albumin as standard. Bile salt concentrations were determined by an enzymatic fluorometric assay (Sterognost-Flu; Nyegaard & Co., Oslo, Norway). Levels of triglycerides, cholesterol, cholesteryl ester, and phospholipids were measured in liver homogenates or bile after lipid extraction, according to Bligh and Dyer³⁵ and as described by Kuipers et al.²⁹ Plasma cholesterol and

triglyceride levels were measured using a commercially available kit (Boehringer Mannheim). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and total bilirubin concentrations in plasma were determined by routine clinical chemistry.

Statistical Analysis

All data are expressed as mean values \pm SEM. Statistical analysis between the experimental groups was performed using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test (simvastatin experiment), or using the unpaired Student *t* test (pravastatin and diosgenin experiments). Level of significance for all statistical analysis was set at $P < 0.05$.

Results

Characteristics of Simvastatin-Treated Rats

We observed no significant differences in body weights or liver/body weight ratios between the control and simvastatin-treated groups (simvastatin-rebound [SR] and simvastatin-continuous [SC]; data not shown). During simvastatin treatment, bile flow was slightly increased from $6.8 \pm 0.9 \mu\text{L} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ in the control group to 8.1 ± 1.0 and $9.0 \pm 1.8 \mu\text{L} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ in the SR and SC groups, respectively, but this did not reach statistical significance. This modest increase may be the result of biliary secretion of simvastatin and/or its metabolites.³⁶ No differences were found in either plasma ALT and AST activities or total bilirubin concentrations between the 3 experimental groups (data not shown).

Effects of Simvastatin on Plasma, Hepatic, and Biliary Lipids

Various parameters of lipid metabolism in control and simvastatin-treated rats are compared in Table 1. Plasma cholesterol concentrations were not affected in SR and SC rats, but plasma triglyceride levels were significantly reduced to 40% and 37%, respectively, of control values. Continuous administration of simvastatin had no effect on the amounts of free cholesterol and cholesteryl ester in the liver. Withdrawal of simvastatin resulted in a significant increase of hepatic cholesteryl ester content to 280% of control values. Hepatic phospholipid and triglyceride levels were not significantly different among the 3 experimental groups. Simvastatin administration had no significant effects on biliary bile salt output. However, a profound effect on the biliary output rates of phospholipids and cholesterol was observed: simvastatin increased the phospholipid content of bile to 174% of control values in the SC group and to 172% in the SR group when expressed relative to bile salt content (Figure

Table 1. Plasma and Hepatic Lipid Levels and Biliary Output Rates in Control and Simvastatin-Treated Rats

	Simvastatin		
	Control	Rebound	Continuous
Plasma (mmol/L)			
Cholesterol	1.45 ± 0.12	1.52 ± 0.11	1.59 ± 0.18
Triglycerides	0.98 ± 0.11	0.40 ± 0.13^a	0.36 ± 0.12^a
Liver (nmol/mg protein)			
Total cholesterol	33.9 ± 2.43	$45.9 \pm 4.05^{a,b}$	34.7 ± 2.36
Free cholesterol	29.7 ± 1.64	37.1 ± 2.90	31.3 ± 1.80
Cholesteryl ester	3.32 ± 0.73	$9.29 \pm 1.18^{a,b}$	2.97 ± 0.62
Triglycerides	38.9 ± 5.40	34.2 ± 7.49	30.3 ± 3.48
Phospholipids	231 ± 7.2	235 ± 6.4	255 ± 6.9
Biliary output (nmol \cdot min ⁻¹ \cdot 100 g body wt ⁻¹)			
Bile salts	200 ± 30	227 ± 11	265 ± 29
Cholesterol	5.7 ± 0.63	$21.5 \pm 3.12^{a,b}$	14.3 ± 1.91^a
Phospholipids	27.7 ± 5.9	53.3 ± 6.5^a	62.9 ± 5.8^a

NOTE. Plasma and liver values represent the average \pm SEM of 10 animals. Biliary output rates represent the average \pm SEM of 3 animals.

^aSignificantly different from controls ($P < 0.01$).

^bSignificantly different from continuous ($P < 0.01$).

1). Biliary cholesterol/bile salt ratios increased to 190% and 335% of control values in the SC and SR groups, respectively, whereas the cholesterol/phospholipid ratio was significantly increased in the SR group only, to 185% of control values (see Figure 1).

Effects of Simvastatin on mRNA Levels of ABC Transport Proteins in Liver

The relative mRNA levels of selected hepatic ABC transport proteins were analyzed by reverse-transcription PCR. We preferred reverse-transcription PCR analysis over Northern blotting because the latter technique is not sensitive enough to detect *mdr1a*, *mdr1b*, and *mrp1* transcripts in the normal rat liver. Also, the specificity of the PCR reactions can be confirmed by sequencing the PCR products. Administration of simvastatin did not result in major changes of *mdr1a*, *spgp*, *mrp1*, and *mrp2* mRNA levels (Figure 2). In contrast, *mdr2* mRNA levels in the SC group were strongly induced but were unaltered in the SR group. *mdr1b* mRNA levels were increased in the SC and, to a lesser extent, in SR groups.

Effects of Simvastatin on Protein Levels of ABC Transporters in Liver

Expression levels of various hepatic ABC transport proteins were examined by Western blot analysis of crude liver membranes and canalicular membrane-enriched fractions. The results of experiment A are shown in Figure 3. All liver samples from the various experimental groups were tested in this way, and similar results were

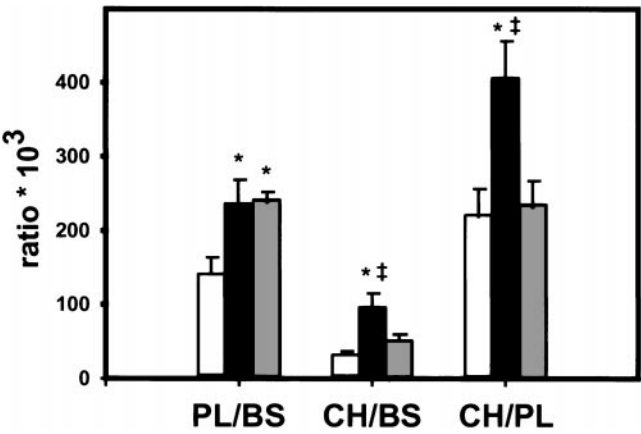


Figure 1. Relative bile salt, phospholipid, and cholesterol contents in bile of control rats and simvastatin-treated rats. Rats were fed standard chow (control; □), simvastatin-containing chow that was replaced 9–11 hours before the end of the experiment (■), or simvastatin-containing chow continuously (▤). Bile was collected for 30 minutes as described in Materials and Methods. Data are presented as ratios $\times 10^3$ of data presented in Table 1 and represent the average \pm SEM of 3 animals. BS, bile salts; CH, cholesterol; PL, phospholipids. * $P < 0.01$, significantly different from control; ‡ $P < 0.01$, significantly different from SC.

obtained ($n = 10$ for each group). Continuous simvastatin administration resulted in pronounced increase of the C219 signal (detecting all Pgps) both in crude membrane pellets (Figure 3) and in canalicular membrane-enriched fractions (data not shown). Pgp levels remained elevated in the SR group. Mdr2 was increased in both SC and SR groups (Figure 3). Spgp protein levels remained unchanged. A slight decrease of mrp2 protein was observed in the SC group. Levels of dipeptidylpeptidase IV (dppIV) protein, used as a canalicular marker protein, were similar in all membrane fractions (data not shown).

Localization of ABC Transporters in Livers of Simvastatin-Treated Rats

Immunohistochemical analysis of liver sections was performed to assess whether the simvastatin-induced expression of *mdr1b* and *mdr2* resulted in changes in the distribution pattern of the encoded Pgps in liver. In control liver, the intensities of P₃II-26 (detecting *mdr2*) and of C219 (detecting all Pgps) signals were stronger in periportal than perivenous regions, although staining of P₃II-26 was weak (Figure 4A and C). Pgps were exclusively found in the canalicular domain of hepatocytes. After simvastatin treatment, however, the P₃II-26 signal was strongly increased, predominantly in periportal areas of the liver lobule (Figure 4B), whereas the induced C219 signal was more homogeneously distributed in treated rat liver (Figure 4D). No differences were observed between SC and SR groups (data not shown). In control liver, spgp, as detected by k12, was uniformly disposed across

the liver acinus; this disposition was not affected by simvastatin administration (Figure 4E and F).

Effects of Pravastatin and Diosgenin on Bile Formation and *mdr* Expression

To study the specificity of the observed induction of *mdr2* and *mdr1b* by simvastatin, experiments were performed with rats that received the hydrophilic HMG-CoA reductase inhibitor pravastatin according to the continuous protocol (pravastatin-continuous [PC]). In addition, another established model of biliary cholesterol hypersecretion, the diosgenin-fed rat, was included in the study. Pravastatin or diosgenin administration did not have significant effects on any of the animal characteris-

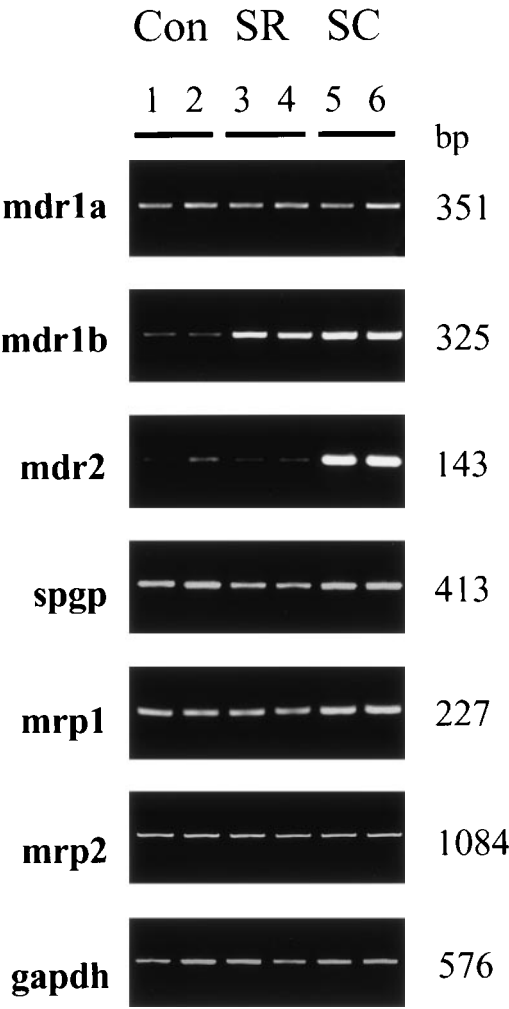


Figure 2. *Mdr1a*, *mdr1b*, *mdr2*, *spgp*, *mrp1*, and *mrp2* mRNA expression during simvastatin treatment. Lanes 1 and 2, control (Con); lanes 3 and 4, simvastatin-rebound (SR); lanes 5 and 6, simvastatin-continuous (SC). mRNA was isolated from control rat liver or simvastatin-treated rat liver, and 2.5 μ g mRNA was transcribed into cDNA and subjected to PCR analysis as described in Materials and Methods. Each band represents the results of a single animal. PCR products are indicated at the left side of each gel, corresponding base pairs at the right side, and the experimental group above the figure. Representative experiment of 5–7/group.

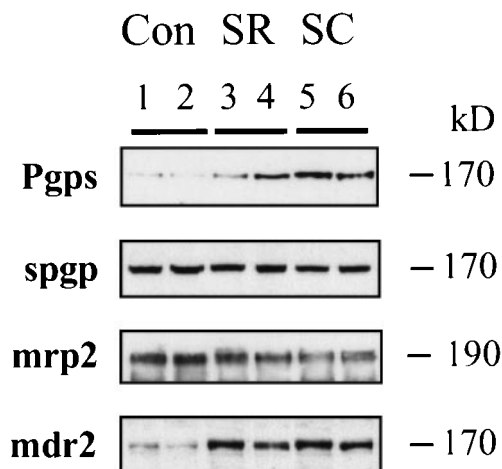


Figure 3. Pggs, spgp, mrp2, and *mdr2* protein levels in membrane fractions of control rat liver and simvastatin-treated rat liver. Lanes 1 and 2, control (Con); lanes 3 and 4, simvastatin-rebound (SR); lanes 5 and 6, simvastatin-continuous (SC). Livers from control rats and simvastatin-treated rats were used for the isolation of crude plasma membranes or canalicular membrane-enriched fractions; 100 μ g of crude membrane proteins or 15 μ g of canalicular membrane-enriched proteins was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Immunoblotting analysis was performed using the primary antibodies C219, k12, k4, and k111, recognizing all Pggs, mrp2, spgp, or *mdr2*, respectively. Bound antibodies were visualized as described in Materials and Methods. For the analysis of Pggs, spgp, and mrp2, crude membrane protein fractions were used. *Mdr2* could only be detected in canalicular membrane protein-enriched fractions. Each band represents the results of a single animal. Transport proteins are indicated at the left side of each blot, apparent molecular weights at the right side, and experimental group above the blot. Representative experiment of 5–7/group.

tics described before. However, during pravastatin treatment, bile flow increased significantly from $7.1 \pm 0.6 \mu\text{L} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ in the control group to $10.0 \pm 0.7 \mu\text{L} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ in the PC group, most likely because of biliary excretion of pravastatin.^{26,37} Diosgenin feeding did not have an effect on bile flow. Plasma lipid levels and biliary lipid output rates of control and pravastatin-treated animals are shown in Table 2. Plasma lipid levels were not significantly affected in the PC group compared with controls. Biliary output rates of bile salts as well as lipids were increased in the PC group compared with controls. However, when expressed relative to bile salt content, the biliary phospholipid and cholesterol contents in the PC group were not significantly increased (Figure 5), although both phospholipid and cholesterol output rates were clearly increased in treated animals (Table 2). In rats treated with diosgenin, biliary cholesterol output was stimulated about 6-fold, whereas phospholipid and bile salt secretion were not affected, as reflected in the marked increases in cholesterol/bile salt and cholesterol/phospholipid ratios (Figure 6). Relative reverse-transcription PCR analysis of cDNA prepared from PC livers revealed an increase in

mdr2 and *mdr1b* mRNA levels (Figure 7), albeit less evidently as observed in SC livers (Figure 2). Diosgenin treatment had no effect on mRNA levels of any of the tested proteins (data not shown).

Discussion

This study shows that statins increase the expression of *mdr2* and *mdr1b* in rat liver. For an effect on *mdr2* expression, a continuous exposure to statins appeared to be necessary, because *mdr2* mRNA levels returned to control levels within 9–12 hours after simvastatin withdrawal. However, during this rebound phase, *mdr2* protein levels remained elevated and, accordingly, biliary phospholipid secretion was increased in both SC and SR groups. In contrast, *mdr1b* mRNA levels remained increased in the SR group, indicating different mechanisms of induction of *mdr2* and *mdr1b* gene expression or differences in mRNA stability.

Statin treatment did not affect plasma cholesterol levels in rats, but simvastatin drastically reduced plasma triglycerides, as expected.⁵ The effect on plasma triglycerides was sustained after withdrawal of simvastatin, probably indicating that hepatic very-low-density lipoprotein production remained suppressed during the time course of this experiment. Neither liver size nor hepatic lipid content was affected by continuous statin feeding. In the simvastatin-rebound group, hepatic cholesterol and cholesteryl ester contents were strongly elevated, probably the result of an inappropriately high synthesis of cholesterol.⁸ It is known that competitive inhibitors of HMG-CoA reductase strongly induce synthesis of HMG-CoA reductase while inhibiting its activity.^{8,38,39}

Simvastatin administration did not affect spgp expression and did not alter biliary bile salt output in either SC or SR groups. We observed a 2-fold increase in phospholipid output in simvastatin-treated animals concomitant with the increase in *mdr2* protein levels. These data clearly support the important role of *mdr2* in the regulation of biliary phospholipid secretion, as initially deduced from studies using mice with a disrupted *mdr2* gene.⁴⁰ Accordingly, biliary phospholipid secretion compared with that of bile salts is increased in mice in which hepatic *mdr2* expression is induced by fibrate feeding⁴¹ and in mice overexpressing *MDR3*, the human homologue of *mdr2*.⁴² The finding that biliary cholesterol/phospholipid ratios in SC and control groups are identical, despite suppression of cholesterol synthesis in the first group, suggests that phospholipid secretion per se is an important regulatory factor of cholesterol secretion. On the other hand, in the SR group cholesterol secretion is markedly enhanced relative to both bile salts and phospholipids. This indicates that, in this condition with

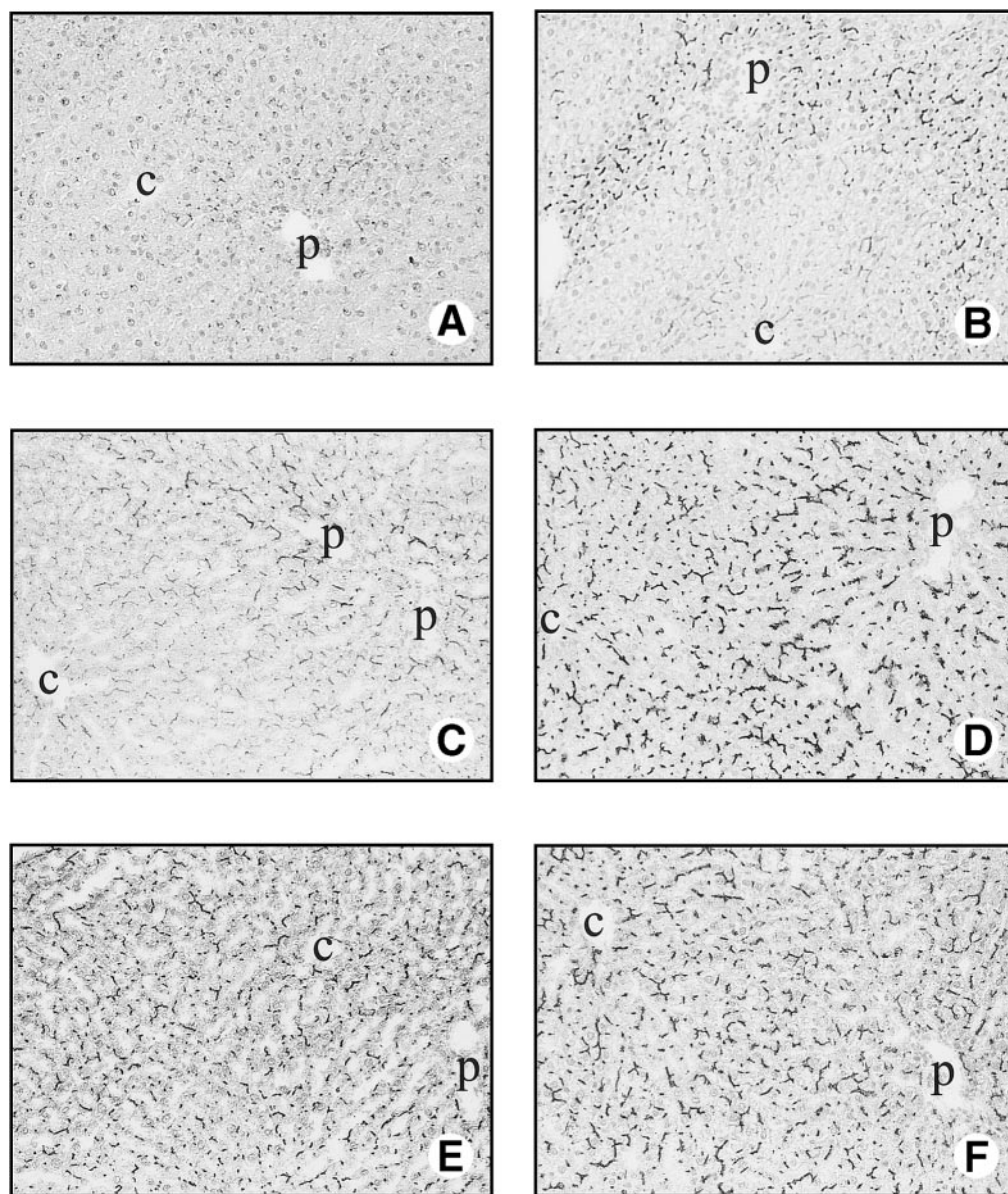


Figure 4. Immunohistochemical localization of (A and B) *mdr2*, (C and D) C219, and (E and F) spgp in (A, C, and E) control rat liver and (B, D, and F) simvastatin-treated rat liver. Frozen liver sections were stained with primary antibodies directed against *mdr2*, all Pggs, and spgp using P₃II-26, C219, and k12, respectively. In normal rat liver, (A) staining of *mdr2*, although weak, was slightly stronger in periportal regions, (C) as was the staining of C219. (E) In normal rat liver, spgp protein was uniformly distributed across the liver acinus. (B) In treated liver, *mdr2* staining was predominantly increased in periportal areas. (D) C219 staining of treated rat liver revealed a homogeneous staining throughout the liver lobule, which was increased compared with control rat liver. (F) In treated rat liver, spgp staining was not affected compared with control rat liver. c, central area; p, portal area. Typical staining patterns of 4–6/group (original magnification 20 \times).

an abrupt increase in *de novo* synthesis of cholesterol, the availability of excess cholesterol in the liver acts as an independent regulator of biliary cholesterol secretion.

To determine the specificity of the observed induction of *mdr2* and *mdr1b* mRNA, the effects of pravastatin administration on expression of these genes was also studied. In addition, a group of rats was fed diosgenin, representing another model of biliary cholesterol hypersecretion. In contrast to simvastatin, pravastatin treatment did not affect plasma triglycerides, indicating that, at the dosage used, simvastatin is a more potent inhibitor of sterol synthesis than pravastatin.^{43,44} As a result, no profound changes in biliary lipid secretion compared with that of bile salts were observed, although there is a tendency toward stimulated biliary lipid secretion in PC animals. Also, a clear increase in *mdr2* and *mdr1b* mRNA levels was detected in pravastatin-treated rats, suggesting

similar cellular actions of simvastatin and pravastatin that are mediated via shared pathways. Data from diosgenin-treated rats demonstrate that hypersecretion of cholesterol can occur independently of *mdr2* induction and that cholesterol hypersecretion per se does not cause induction of *mdr2*.

In conclusion, our data suggest that the induction of *mdr2* expression, and, consequently, of biliary phospholipid secretion in statin-treated rats gives rise to a proportional increase in cholesterol secretion. Cessation of statin treatment results in an additional stimulation of biliary cholesterol secretion that may be related to rapid expansion of the intracellular cholesterol pool because of a sudden increase in cholesterol synthesis.

Statins cause a transient state of cellular free cholesterol deprivation, resulting in enhanced transcription of sterol-regulated genes,⁴⁵ mediated by sterol regulatory element–

Table 2. Plasma and Biliary Output Rates in Control and Pravastatin-Treated Rats

	Pravastatin	
	Control	Continuous
Plasma (mmol/L)		
Cholesterol	1.95 ± 0.07	1.78 ± 0.12
Triglycerides	0.51 ± 0.06	0.46 ± 0.05
Biliary output (nmol · min ⁻¹ · 100 g body wt ⁻¹)		
Bile salts	256 ± 32	361 ± 39
Cholesterol	4.6 ± 0.49	7.9 ± 0.87 ^a
Phospholipids	37.7 ± 3.0	62.7 ± 6.5 ^a

NOTE. Plasma values represent the average ± SEM of 3 control and 5 pravastatin-treated animals. Biliary output rates represent the average ± SEM.

^aSignificantly different from controls ($P < 0.05$).

binding proteins (SREBPs).⁴⁶ SREBPs activate transcription by binding to sterol regulatory elements (SREs) in the promoter regions of target genes.⁴⁶ Importantly, SREBP-1 is down-regulated and SREBP-2 is up-regulated in livers of hamsters and mice during treatment with lovastatin and a bile salt-binding resin.^{47,48} We hypothesize that transcriptional control of *mdr2* gene expression might, at least partially, be mediated via SREBPs. The 5'-flanking region of the *mdr2* gene⁴⁹ contains elements that are possibly recognized by SREBPs: an inverted motif that is homologous to SRE-3⁵⁰ (position -278 to -269) and an inverted SRE-1 half-site (position -193 to -188).⁵¹

The *mdr2*-positive cells were mainly localized to periportal regions of control livers; this was also true in livers from simvastatin-treated animals. This zonal distribution is very similar to the reported distribution of HMG-CoA reductase and HMG-CoA synthase before

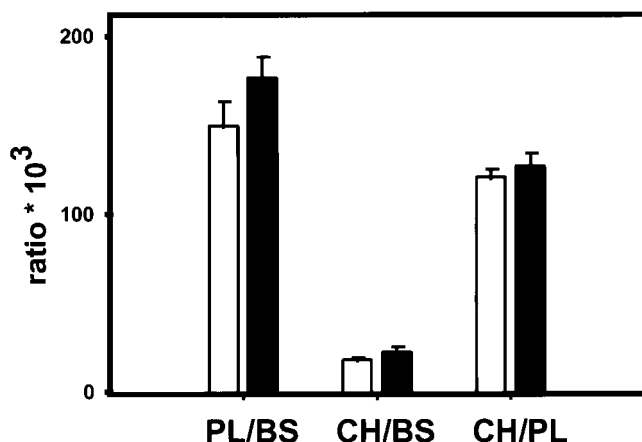


Figure 5. Relative bile salt, phospholipid, and cholesterol contents in bile of control rats and pravastatin-treated rats. Rats were fed standard chow (□) or pravastatin-containing chow (■). Bile was collected for 30 minutes as described in Materials and Methods. Data are presented as ratios × 10³ and represent the average ± SEM of 3 control and 5 pravastatin-treated animals. BS, bile salts; CH, cholesterol; PL, phospholipids.

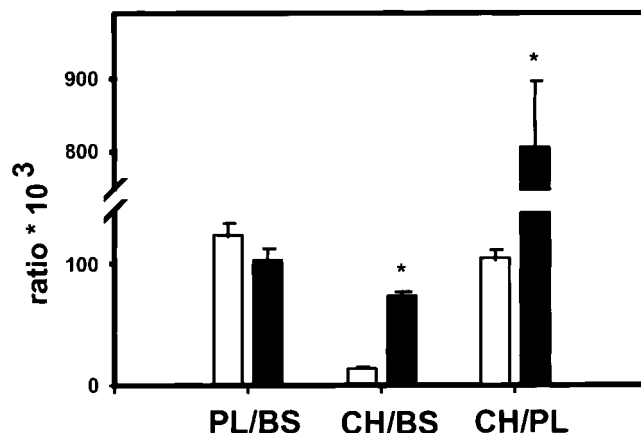


Figure 6. Relative bile salt, phospholipid, and cholesterol contents in bile of control rats and diosgenin-treated rats. Rats were fed standard chow (□) or diosgenin-containing chow (■). Bile was collected for 30 minutes as described in Materials and Methods. Data are presented as ratios × 10³ and represent the average ± SEM of 3 animals. Note the axis break. BS, bile salts; CH, cholesterol; PL, phospholipids. * $P < 0.001$, significantly different from control.

and after statin treatment,^{38,52} which suggests that the factors controlling the expression of *mdr2* and these enzymes may be similar. Staining with C219 of control rat liver revealed a similar heterogeneous staining pattern as observed for P₃II-26. However, unlike P₃II-26, the C219 signal was more homogeneously distributed in simvastatin-treated animals. Because the spgp staining is unchanged and the *mdr2* staining is predominantly increased in periportal regions after simvastatin treatment, we conclude that this may, at least partially, be the result of increased expression of *mdr1b* Pgp.

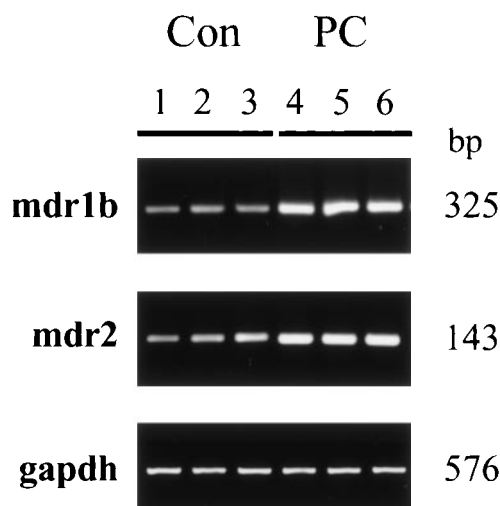


Figure 7. *Mdr1b* and *mdr2* mRNA expression during pravastatin treatment. Lanes 1–3, control (Con); lanes 4–6, pravastatin-continuous (PC). mRNA was isolated from control rat liver or pravastatin-treated rat liver; 2.5 µg mRNA was transcribed into cDNA and subjected to PCR analysis as described in Materials and Methods. Each band represents the results of a single animal. PCR products are indicated at the left side of each gel, corresponding base pairs at the right side, and experimental group above the figure. Representative experiment of 3–5/group.

Mdr1b mRNA levels also respond to statin administration, although this response is not as dramatic as observed in other experimental conditions.^{53–55} The induction of *mdr1b* mRNA persists after cessation of simvastatin treatment, in contrast to the situation observed for *mdr2*. This may be the result of a longer half-life of *mdr1b* mRNA, enhanced transcription rate, or a differential regulation of the expression of these 2 genes,^{12,53,54} which is in accordance with the different physiological functions of these proteins. In rat liver, a direct induction of *mdr1b* gene expression has been shown as a result of exposure to various xenobiotics,^{12,56,57} but the mechanism by which *mdr1b* mRNA is induced remains to be determined. Yamazaki et al.²⁶ recently provided evidence that the biliary excretion of the relatively hydrophilic statin pravastatin is predominantly mediated by *mrp2*. Whether *mrp2* is also involved in the biliary excretion of simvastatin or its metabolites is not known. Because of their hydrophobic nature, *mdr1a/1b* may also be candidate transport proteins.

In conclusion, we have shown that during treatment of rats with the HMG-CoA reductase inhibitors simvastatin and pravastatin, the expression of *mdr2* and of *mdr1b* is enhanced, revealing a novel, until now unrecognized effect of these cholesterol-lowering agents. Increased *mdr2* expression may facilitate removal of excess cholesterol that enters the liver as a consequence of induced LDL receptor activity during statin treatment. In addition, these findings may provide clues for mechanisms involved in regulation of Pgp expression and function in rat liver.

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